Endocytosis of Prion Protein Is Required for ERK1/2 Signaling Induced by Stress-Inducible Protein 1

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The secreted cochaperone STI1 triggers activation of protein kinase A (PKA) and ERK1/2 signaling by interacting with the cellular prion (PrP^C) at the cell surface, resulting in neuroprotection and increased neuritogenesis. Here, we investigated whether STI1 triggers PrP^C trafficking and tested whether this process controls PrP^C-dependent signaling. We found that STI1, but not a STI1 mutant unable to bind PrP^C, induced PrP^C endocytosis. STI1-induced signaling did not occur in cells devoid of endogenous PrP^C; however, heterologous expression of PrP^C reconstituted both PKA and ERK1/2 activation. In contrast, a PrP^C mutant lacking endocytic activity was unable to promote ERK1/2 activation induced by STI1, whereas it reconstituted PKA activity in the same condition, suggesting a key role of endocytosis in the former process. The activation of ERK1/2 by STI1 was transient and appeared to depend on the interaction of the two proteins at the cell surface or shortly after internalization. Moreover, inhibition of dynamin activity by expression of a dominant-negative mutant caused the accumulation and colocalization of these proteins at the plasma membrane, suggesting that both proteins use a dynamin-dependent internalization pathway. These results show that PrP^C endocytosis is a necessary step to modulate STI1-dependent ERK1/2 signaling involved in neuritogenesis.

Key words: neurodegeneration; endocytosis; clathrin; raft; prion diseases; flotillin; ERK

Introduction

Prions are believed to be the causal agent of transmissible spongiform encephalopathies that affect humans and other species. These neurological disorders have in common the corruption of a glycosylphosphatidylinositol (GPI)-anchored host protein known as the prion protein (PrP^C). It is widely accepted that a misfolded conformer of PrP^C, PrP^{Sc} (here to denote infectious, β sheet-enriched prion), is the major component of the infectious

particle (Prusiner, 1998; Weissmann, 1999). PrP^{Sc} interacts with PrP^C at the cell surface or along the endocytic pathway (Caughey and Raymond, 1991; Caughey and Baron, 2006), but how this interaction imprints novel information to cause disease in the host is a matter of debate. The proposal that PrP^{Sc} represents a gain-of-function, toxic conformer has received much attention, whereas the possibility that alterations in physiological functions of PrP^C contribute to the disease has only recently started to be addressed (Samaia and Brentani, 1998; Martins et al., 2002; Linden et al., 2008).

One of the major difficulties has been to attribute defined physiological roles for PrP^C. Nonetheless, studies in yeast, mammalian cells, and mice models support the hypothesis that PrP^C plays a major role in neuroprotection (for review, see Westergaard et al., 2007). Recent data pointed at specific domains of the molecule that are essential for PrP^C-mediated protection. Mice expressing a PrP^C mutant without amino acids 105–125 (hydrophobic domain) in a PrP^C-null background spontaneously developed severe neurodegenerative illness that was lethal within 1 week after birth (Li et al., 2007). Additionally, the presence of a PrP^C variant lacking residues 94–134 induced a rapidly progressive lethal phenotype with extensive central and peripheral myelin degeneration (Baumann et al., 2007).

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We have demonstrated that the PrP^C domain, which includes amino acids 113–128, is the binding site for the cochaperone stress-inducible phosphoprotein 1 (STI1). PrP^C engagement with STI1 rescued retinal and hippocampal neurons from programmed cell death through activation of protein kinase A (PKA) (Chiarini et al., 2002; Zanata et al., 2002; Lopes et al., 2005). Additionally, PrP^C–STI1 binding also induced the differentiation of hippocampal neurons by ERK1/2 activation (Lopes et al., 2005). Understanding the fate of both STI1 and PrP^C after their association at the cell surface may help clarify the mechanisms associated with the neurotrophic roles of PrP^C, and its possible bearing on loss-of-function components of prion diseases.

To address these questions, we initially tested whether STI1 alters PrP^C trafficking. Indeed, we found that interaction of these proteins at the cell surface triggered PrP^C endocytosis. Remarkably, the transient ERK1/2 activity induced by PrP^C interaction with either recombinant STI1 or endogenous STI1 secreted by astrocytes depended on PrP^C endocytosis, whereas activation of PKA was not affected when PrP^C trafficking was impaired. STI1 localized in PrP^C-positive organelles only in the initial periods of internalization, suggesting that signaling is triggered by the interaction of these two proteins at the cell surface or shortly after. These data show for the first time that ligand-induced endocytosis of PrP^C is important for cellular signaling.

Materials and Methods

Animals

The *Principles of Laboratory Animal Care* (National Institutes of Health publication number 85-23, 1996) was strictly followed in all experiments. Zrch1 *Prnp*^{0/0} mice were provided by Dr. C. Weissmann (Scripps Florida, Jupiter, FL) (Bueler et al., 1992), and the wild-type control mice (Zrch1 *Prnp*^{+/+}) were generated by crossing F1 descendants from 129/SV and C57BL/6J mating.

Cell culture

SN56 cells are derived from mouse septum neurons (Hammond et al., 1990) and were cultured as described previously (Santos et al., 2001). CF-10, a PrP^C-null neuronal cell line derived from 129/Ola *Prnp*^{0/0} mice (Manson et al., 1994) and positive for the neuroectodermal stem cell marker nestin (I. Vorberg and S. A. Priola, unpublished observation), was used for reconstitution of PrP^C signaling. CF-10 cells were cultured in OptiMEM (Invitrogen) containing glutamine (2 mM; Invitrogen), penicillin (100 IU), and streptomycin (100 µg/ml; Invitrogen) supplemented with 10% fetal bovine serum.

Primary hippocampal cultures were obtained from embryonic day 17 brains of either wild-type (*Prnp*^{+/+}) or *Prnp*^{0/0} mice (Bueler et al., 1992). The hippocampus was aseptically dissected in HBSS (Invitrogen) and treated with trypsin (0.06%) in HBSS for 20 min at 37°C. The protease was inactivated with 10% FCS in Neurobasal medium (Invitrogen) for 5 min. After three washes with HBSS, cells were mechanically dissociated in Neurobasal medium containing B-27 supplement (Invitrogen), glutamine (2 mM; Invitrogen), penicillin (100 IU), and streptomycin (100 µg/ml; Invitrogen). Cells (0.5 × 10⁶ cells) were plated onto coverslips (22 mm) coated with 5 µg/ml poly-L-lysine (Sigma).

Plasmids

The GFP-PrP^C vector, GFP-Rab5, dynamin I, the dominant-negative dynamin I K44A, and GFP-Rab7 mutant plasmids have been described previously (Lee et al., 2001; Santos et al., 2001; Barbosa et al., 2002; Magalhães et al., 2002, 2005; Ribeiro et al., 2005). Flotillin1–green fluorescent protein (GFP) and caveolin-1–GFP were kindly provided by Dr. B. J. Nichols (MRC Laboratory of Molecular Biology, Cambridge, UK). The 3F4-tagged plasmids, PrP3F4, and the N-PrP3F4 (N-terminally mutated, altered from ²³KKRPKP²⁸ to ²³KQHPS²⁸) (Sunyach et al., 2003) were kindly provided by R. Morris (Wolfson Centre for Age Related Diseases, Guy's Hospital Campus, King's College, London, UK). It is important to note that in the N-PrP3F4 mutant, a serine is present at the

position 27 and not histidine as published previously (Sunyach et al., 2003) (R. Morris, personal communication).

Transfection of cell lines and isolation of PrP^C-expressing cells

SN56 cells were plated on coverslips for 2 d and transfected using the liposome-mediated method (Effectene; Qiagen) according to the manufacturer's instruction using a 1:10 ratio of DNA to Effectene. After 6 h of transfection, cells were differentiated in serum-free medium supplemented with 1 mM dibutyryl-cAMP (Sigma) for 2 or 3 d with medium changes every day. In cotransfection experiments, a total of 3–5 µg of DNA was used with a plasmid ratio of 1:4 of clathrin–GFP, flotillin1–GFP, and caveolin-1–GFP and a ratio of 1:2 for GFP-PrP^C and dynamin I or dynamin I K44A.

CF-10 cells were transfected with either 3F4-tagged PrP^C (PrP3F4) or the mutant PrP^C N-PrP3F4 (Sunyach et al., 2003) using a liposome-mediated method (Lipofectamine 2000; Invitrogen) according to the manufacturer's instructions using a 1:3 ratio of DNA to lipofectamine. After transfection, the cells were selected with G418 (2 mg/ml; Invitrogen) for 15 d, and resistant cells (10⁷) were incubated with mouse anti-PrP^C serum (Zanata et al., 2002) at a 1:100 dilution for 1 h at 4°C. As a negative control, the cells were incubated with preimmune serum. After three washes with PBS, cells were incubated with anti-mouse IgG R-phycoerythrin conjugated at a 1:200 dilution for 1 h at 4°C. Cell sorting was performed using a FACSCalibur flow cytometer (BD Biosciences). Fluorescence was measured using a 488 nm argon laser and FL2-H channel (red fluorescence, 585/42 nm), and data acquisition from 10,000 events was analyzed using CellQuest software (BD Biosciences). Sorting was accomplished using logical gating of the cells in the forward scatter (FSC) versus side scatter and the FSC versus FL2 dot plots. Only events that entered a gate consisting of both cell regions were sorted.

Alexa Fluor 594, 568, or 488 protein labeling

Recombinant STI1 [wild type or deleted in residues 230–245 (STI1_{Δ230–245})] was expressed and purified as described previously (Zanata et al., 2002). STI1 labeling was performed using an Alexa Fluor 594 (AF594), Alexa Fluor 568 (AF568), or Alexa Fluor 488 (AF488) labeling kit (Invitrogen). Briefly, STI1 (2 mg/ml) diluted in PBS containing 100 mM sodium bicarbonate was labeled with the reactive dye for 1 h at room temperature, and free dye was separated from the labeled protein by size exclusion chromatography using a PD-10 column (GE Healthcare). Protein concentration was determined with Bradford reagent (Bio-Rad).

Protein detection

Fluorescent-labeled proteins (STI1 and STI1_{Δ230–245}; 2 µg) were submitted to 10% SDS-PAGE. To visualize labeled proteins, the gel was irradiated with ultraviolet light, and images were acquired using a UV transilluminator MultiDoc-It-Digital Imaging System (Bioimaging Systems). Alternatively, proteins resolved in 10% SDS-PAGE were subjected to immunoblotting with polyclonal antibody anti-STI1 (1:10,000) (Zanata et al., 2002). Rabbit nonimmune-purified IgG was used as the immunoblotting negative control. For detection of PrP^C, cell extracts were prepared by homogenizing the cell pellet in lysis buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% acid deoxycholic) and a protease inhibitor mixture (complete protease inhibitor tablets; Roche Diagnostics) or a protease inhibitor mixture (Sigma) at twice the concentration suggested by the manufacturers. Proteins from cells were resolved in SDS-PAGE, transferred to nitrocellulose or Immobilon-P transfer membranes (Millipore), and incubated with mouse anti-PrP^C antibody (Zanata et al., 2002). Staining was revealed by enhanced chemiluminescence (ECL Plus; GE Healthcare) or Super Signal Chemiluminescent Substrate (Pierce).

Fluorescence imaging

Confocal microscopy was performed using a Bio-Rad MRC 1024 laser-scanning confocal system running the Lasersharp 3.0 software coupled to a Zeiss microscope (Axiovert 100) with a 100 × 1.4 numerical aperture (NA) or 63 × 1.3 NA oil-immersion lens (Zeiss), a Bio-Rad Radiance 2100 laser-scanning confocal system coupled to a Nikon microscope (TE2000-U), and a Leica SP5 laser-scanning confocal microscope using a

Figure 1. *A, B*, STI1 induces PrP^C internalization. SN56 cells expressing GFP-PrP^C were treated with 1 μM STI1 (*A*) or STI1_{Δ230–245} (*B*) for 45 min at 37°C. The left and right panels show the green fluorescence from GFP-PrP^C before and after the incubation with STI1 or STI1_{Δ230–245}, respectively. *A* and *B* represent Z projections acquired before and after the perfusion. Images are representative of nine and five independent experiments with multiple culture plates in which 40 and 12 cells were analyzed. Scale bars, 20 μm. *C*, Flow cytometry assay from SN56 cells treated with 500 μM Cu²⁺, 1 μM wild-type STI1, or 1 μM STI1_{Δ230–245} for 40 min. Cells were incubated with nonimmune or anti-PrP^C mouse serum followed by R-phycoerythrin-labeled anti-mouse IgG. *D*, The fluorescence for cell-surface PrP^C in untreated cells as described in *C* was set up to 100%, and the levels of PrP^C cell-surface expression after treatment with the indicated concentrations of Cu²⁺, STI1, or STI1_{Δ230–245} were normalized to untreated cells. The results shown are the mean values of six independent experiments. Error bars represent SEM. ANOVA followed by Tukey's HSD test was used for comparisons. *p < 0.05.

63 × 1.2 NA water-immersion or a 63 × 1.4 oil-immersion lens (Leica). Cells on coverslips were washed and maintained in serum-free medium or Krebs–Ringer–HEPES (KRH) buffer (124 mM NaCl, 4 mM KCl, 1.2 mM MgSO₄, 25 mM HEPES, 10 mM glucose, and 1 mM CaCl₂) during image acquisition. In live cell experiments, a FCS2 chamber and objective heater system (Biopatch) were used to maintain cells at 37°C. Image analysis and processing were performed with Lasersharp (Bio-Rad), Confocal Assistant, Adobe Photoshop, Metamorph, Leica Application Suite Advanced Fluorescent Lite, and ImageJ (version 1.24) software.

Internalization of STI1 and PrP^C

SN56 cells were incubated with 1 μM fluorescent STI1 for different periods at 37°C in 5% CO₂, washed three times with serum-free medium, and visualized by confocal microscopy. For competition assays, cells were incubated with 10 μM STI1 or albumin in DMEM for 1 h at 4°C, followed by 1 μM fluorescent STI1. The coverslips were washed with serum-free medium and visualized by confocal microscopy.

Cells expressing GFP-PrP^C or coexpressing GFP-PrP^C and dynamin I K44A were maintained in a FCS2 chamber at 37°C and perfused with KRH to obtain the first image. After that, cells were perfused with 1 μM fluorescent STI1 or STI1_{Δ230–245}, and additional optical sections were acquired each minute for 50 min.

For cell-surface labeling, CF-10 PrP3F4 or N-PrP3F4 cells were treated with STI1 or STI1_{Δ230–245}, as described in the figure legends, for 20 min, washed in PBS three times, and fixed in 3.5% paraformaldehyde without any detergent for 20 min. Cells were incubated simultaneously with monoclonal 3F4 antibodies (1:100; Dako), and after washing with PBS, cells were incubated goat anti-mouse AF488 secondary antibodies (Invitrogen), mounted in coverslips with Hydromount (National Diagnostics), and imaged with the SP5 confocal microscope. In these experiments, nuclei were stained with Syto60Red (Invitrogen).

Biotinylation of cell-surface proteins was performed as described previously (Ribeiro et al., 2005; Lee et al., 2007). Briefly, cells were incubated with STI1 or 500 μM Cu²⁺ for 5 or 10 min, respectively, transferred to ice, washed, and incubated on ice in PBS/CM (PBS supplemented with 1.0 mM MgCl₂ and 0.1 mM CaCl₂). Cell-surface proteins were biotinylated with 1 mg/ml sulfo-NHS-SS-biotin (Pierce) for 1 h on ice. To

quench the biotinylation reaction, cells were washed and incubated for 30 min with cold 100 mM glycine in PBS/CM, followed by three washes with cold PBS/CM, and proteins were extracted using 10 mM Tris, pH 7.8, 0.1 M NaCl, 10 mM EDTA, 0.5% Triton X-100, and 0.5% acid deoxycholic. Biotinylated proteins were separated from nonbiotinylated proteins by Neutravidin bead pull-down from equivalent amounts of total cellular protein (800 μg) from each sample. The biotinylated proteins were subjected to SDS-PAGE, followed by electroblotting onto polyvinylidene fluoride membranes, and revealed using a mouse anti-PrP^C antibody (Zanata et al., 2002). For quantification, the major glycosylated band of PrP^C in nonsaturated blots was analyzed using ImageQuant TL and normalized by the expression of PrP^C in the lysates.

Labeling of organelles

Labeling of endosomes was performed by incubating cells with 40 μg/ml AF488-labeled transferrin (Invitrogen) at 37°C in 5% CO₂ for 40 min. After incubation, cells were washed three times with PBS and fixed with 3% paraformaldehyde in PBS for 20 min for posterior imaging. Labeling of late endosomes/lysosomes was done by incubating cells with 1 μM LysoSensor Green DND-189 (Invitrogen) at 37°C in 5% CO₂ for 1 h, and cells were washed as described above and imaged.

Quantification of fluorescence

The effect of dynamin K44A expression on STI1 internalization was evaluated using the ImageJ software or MetaMorph. The total fluorescence inside cells after a 40 min incubation with STI1-AF568 was quantified. Images were thresholded, and the total fluorescence was detected automatically and independently by the software. The results were expressed as the mean of total fluorescence per cell. For colocalization indices, cells were analyzed using MetaMorph, by independently counting fluorescent objects (vesicles) and analyzing the percentage of colocalization independently by the software.

Conditioned medium from astrocytes

Primary mouse astrocyte cultures were obtained as described previously (Lima et al., 2007). After reaching confluence, cells were maintained in DMEM without serum for 48 h. The conditioned medium (CM) was collected, centrifuged for 10 min to remove cellular debris, and filtered in 0.2 μm membranes. CM (total volume of 30 ml) was concentrated to a final volume of 150 μl (200×) in Minicon Static concentrator B12 (Millipore). A total of 30 μl of the 200× concentrated CM was used to measure STI1 concentration. Alternatively, 50 μl of the 200× concentrated CM was immunodepleted of STI1 using a rabbit anti-STI1 antibody (IgG, 4 μg/ml) overnight at 4°C (Lima et al., 2007), mixed with protein A-Sepharose for 2 h at 4°C, and centrifuged. The pellets (washed three times) and supernatants were analyzed for the presence of STI1.

Kinase assays

P44/42 extracellular signal-regulated kinase phosphorylation. Phosphorylation assays were performed using the PhosphoPlus p44–42 extracellular signal-regulated kinase (ERK) (Thr202/Tyr204) antibody kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, CF-10 cell lines (5 × 10⁴ cells, serum starved for 48 h with medium change every 24 h) and hippocampal primary culture (10⁶ cells) were stimulated or not with recombinant STI1 (0.5 μM) or 50 μl of the 200× concentrated CM to a final volume of 1 ml (5 nM STI1), rinsed once with ice-cold PBS, and lysed in Laemmli buffer. Cell extracts were subject to SDS-PAGE, followed by immunoblotting with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies (Cell Signaling Technology). The bands obtained after x-ray film exposure to the membranes were analyzed by densitometric scanning and quantified using the Scion Image software.

Figure 2. Internalization of PrP^C is dependent on the N-terminal basic motif. The PrP^C-null cell line CF-10 was transfected with an expression vector encoding wild-type PrP^C (PrP3F4) or a mutated PrP^C (N-PrP3F4) protein, the internalization of which is impaired, and stably transfected cells were sorted. **A**, Flow cytometry of nonpermeabilized cells detected using anti-PrP^C antibodies (except in CF-10 cells, black lines that were incubated only with secondary antibodies). **B**, Western blot assays using anti-PrP^C antibodies show similar expression of ectopic proteins. **C**, PrP3F4 cells were kept in KRH or treated with 2 μ M STI1 $_{\Delta 230-245}$ (second column) or STI1 (third column) for 20 min at 37°C. After treatment, cells were fixed and immunostained for cell-surface PrP^C using the 3F4 antibody. **D**, As in **C**, the N-PrP3F4 mutant cells were treated with 2 μ M STI1 for 20 min at 37°C. Scale bars, 20 μ m. **E**, PrP3F4- or N-PrP3F4-expressing cells were treated with STI1 or KRH for 5 min and iced. Cell-surface PrP^C after these treatments was detected by biotinylation of cell-surface proteins. Biotinylated proteins were isolated using Neutravidin beads, subjected to SDS-PAGE, and immunoblotted using a mouse anti-PrP^C antibody. The lysates represent the expression of PrP3F4 or N-PrP3F4 proteins, and beads represent biotinylated cell-surface PrP^C after treatment with STI1 or KRH (control). Note that STI1 decreased the amount of PrP3F4 in the membrane in the two lanes (duplicates) labeled STI1 compared with control, whereas N-PrP3F4 was not decreased. Treatment with 500 μ M CuSO₄ for 10 min was used to test for efficient detection PrP^C cell-surface sequestration. The blots are representative of six or seven experiments, respectively.

PKA activation. Primary hippocampal neurons (10⁶ cells), the SN56 cell line (10⁶ cells, medium starved 24 h), or the CF-10 cell line (10⁵ cells, serum starved for 48 h with medium change every 24 h) were preincubated with 100 μ M IBMX (Sigma) for 1 h at 37°C and 5% CO₂ and treated with STI1 (1 μ M) or forskolin (10 μ M) for 20 min at 37°C. The cells were washed with PBS and homogenized with ice-cold extraction buffer (150 mM NaCl, 20 mM MgCl₂, 1% Triton X-100, and 25 mM Tris-HCl, pH 7.4) plus Complete Protease Inhibitor Cocktail (Roche). Cellular debris was removed by centrifugation at 6000 $\times$ g for 10 min. The PKA activity was determined by γ [P³²]-ATP incorporation to a PKA-specific substrate provided by the PKA assay system kit (Millipore). The reaction was performed according to the manufacturer's instructions.

Flow cytometry assay

SN56 cells were preincubated with blocking solution (0.5% BSA in PBS) in the absence or presence of STI1 or copper sulfate for

20 or 40 min at 37°C. Cells were washed and incubated with an anti-PrP^C antibody (1:100) (Zanata et al., 2002), followed by anti-mouse IgG conjugated to R-phycoerythrin (1:200; Dako), both for 1 h at 4°C. Analyses were performed using a FACSCalibur flow cytometer (BD Biosciences), and data acquisition from 10,000 events was analyzed using CellQuest software (BD Biosciences).

Statistical analysis

The mean values of at least three independent datasets are shown in the figures; the error bars represent SEM. ANOVA followed by Tukey's honestly significant difference (HSD) test or Kruskal–Wallis one-way ANOVA followed by a Dunn's *post hoc* test were used for multiple comparisons. For all tests, results were considered statistically significant when *p* was <0.05.

Figure 3. PrP^C endocytic trafficking is necessary for STI1-PrP^C-dependent ERK1/2 but not for PKA activation. **A**, Cells were treated with forskolin or 1 μ M of STI1, and the PKA activity was evaluated. **B**, Cells were treated with 0.5 μ M STI1 for 1 or 5 min, and ERK1/2 activity (pErk) was analyzed. **C**, Cells were treated with 0.5 μ M STI1 $\Delta_{230-245}$ for 1 or 5 min, and ERK1/2 activity was analyzed. The basal activity of CF-10 cells without treatment was normalized to 1, and the other values are relative to it. The results show the mean values of five (**A**) or four (**B**, **C**) independent datasets. Error bars represent SEM. ANOVA followed by Tukey's HSD test was used for comparisons. * $p < 0.05$. A.U., Arbitrary units.

Results

STI1 induces PrP^C internalization

To test whether interaction with STI1 causes any consequence for PrP^C localization in living cells, we expressed GFP-PrP^C ectopi-

Figure 4. STI1 secreted by astrocytes induces ERK1/2 activation. **A**, Increasing amounts of recombinant STI1 (from 10 to 500 ng) and 30 μ l of 200 $\times$ concentrated CM was loaded onto SDS-PAGE. Immunoblotting was developed with anti-STI1 antibodies. **B**, STI1 was immunoprecipitated from 150 μ l of concentrated CM. The supernatant and the pellet were resolved by SDS-PAGE and detected with anti-STI1 antibodies: lane 1, 30 μ l of concentrated CM; lane 2, 30 μ l of immunodepleted concentrated CM; lane 3, 30 μ l pellet from immunoprecipitation. **C**, CF-10 or CF-10-expressing PrP3F4 were treated with 50 μ l of concentrated CM (STI1 final concentration of 5 nM) or immunodepleted concentrated CM for 1 or 5 min, and ERK1/2 activity was analyzed. The basal activity of CF-10 cells without treatment was normalized to 1, and the other values are relative to it. The results show the mean values of three independent datasets. Error bars represent SEM. ANOVA followed by Tukey's HSD test was used for comparisons. * $p < 0.05$. A.U., Arbitrary units.

cally. This fluorescent protein has been previously shown to respond to Cu²⁺ and to present identical localization as endogenous PrP^C. For these experiments, we used SN56 cells, in which the cellular trafficking of fluorescent PrP^C and PrP^{res} was studied and which have also been shown to be infected by PrP^{res} (Lee et al., 2001; Magalhães et al., 2005). Maximum projection images obtained from confocal stacks (data not shown) demonstrated that, as described previously (Lee et al., 2001), GFP-PrP^C is present at the cell surface and in the perinuclear region that represents the Golgi apparatus and endosomes (Magalhães et al., 2002). In control experiments, we reproduced the observation that Cu²⁺ evokes internalization of GFP-PrP^C (data not shown) (Lee et al., 2001; Magalhães et al., 2002). We then evaluated GFP-PrP^C distribution in SN56 cells after treatment with STI1 or a deletion mutant unable to bind PrP^C, STI1 $\Delta_{230-245}$ (Lopes et al.,

2005). We noted that a significant fraction of the green fluorescence representing PrP^C disappeared from the cell surface and accumulated inside cells after STI1 addition (Fig. 1A). Conversely, GFP-PrP^C was not internalized in cells similarly treated with STI1 $_{\Delta 230-245}$ (Fig. 1B), indicating the need of STI1–PrP^C interaction for PrP^C endocytosis.

To test whether endogenous PrP^C was also internalized in cells in response to STI1 and to quantify this effect, flow cytometry assays were performed. SN56 cells express PrP^C (Lee et al., 2001; Magalhães et al., 2005; Baron et al., 2006), and treatment with 500 μ M Cu²⁺ evoked sequestration of cell-surface PrP^C (Fig. 1C). STI1 also induced internalization of endogenous PrP^C in a dose-dependent manner (Fig. 1D), whereas STI1 $_{\Delta 230-245}$ was unable to promote endocytosis of PrP^C (Fig. 1D). The present results show that interaction of STI1 and PrP^C at the cell surface triggers internalization of PrP^C, albeit at lower levels than copper. Control experiments showed that STI1 interaction with PrP^C does not shed the latter from the cell surface, because cultures treated with STI1 showed no increase in PrP^C in CM (data not shown).

Endocytic trafficking of PrP^C is required for STI–PrP^C-dependent ERK1/2 but not for activation of PKA

The endocytic trafficking of membrane receptors is important for attenuating ligand-induced signaling, but it can also be critical to trigger and modulate specific signaling pathways as shown, for example, for epidermal growth factor receptors (Vieira et al., 1996). Our previous data showed that PrP^C–STI1 interaction triggers both PKA and ERK1/2 activation promoting neuronal survival and differentiation, respectively (Chiarini et al., 2002; Lopes et al., 2005). To test for a role of STI1-induced PrP^C endocytic trafficking on these signaling pathways, we expressed either a mutant PrP^C, the internalization of which is impaired (N-PrP3F4), or its wild-type control (PrP3F4) (Sunyach et al., 2003) in PrP^C-null CF-10 neuronal cells. Transfected cells were selected for similar expression of either wild-type PrP3F4 or N-PrP3F4 mutated PrP^C, as verified by flow cytometry (Fig. 2A) and Western blot assays (Fig. 2B). As expected, no endogenous PrP^C expression was present in untransfected CF-10 cells (note that black and red lines represent CF-10 cells incubated without or with PrP^C antibodies, respectively) (Fig. 2A,B).

We initially tested whether in these cells STI1 would also induce endocytosis. Immunofluorescence analysis of cell-surface PrP3F4 indicates that STI1, but not STI1 $_{\Delta 230-245}$, decreased cell-surface immunolabeling for PrP^C (Fig. 2C). In contrast, in N-PrP3F4-expressing cells, treatment with STI1 did not change cell-surface immunolabeling (Fig. 2D). To complement these experiments and quantify the effect of STI1, we used biotinylation of cell-surface PrP^C (Fig. 2E). In these experiments, we measured the amount of PrP3F4 remaining at the cell surface after the cells were treated with STI1 (Ribeiro et al., 2005; Lee et al., 2007). In control experiments, cells exposed to Cu²⁺ showed a decrease in cell-surface PrP^C ($63 \pm 10\%$ of decrease; mean $\pm$ SEM for seven experiments). Treatment of cells with STI1 for 5 min caused sequestration of PrP3F4 with consequent decrease in cell-surface PrP^C (Fig. 2E) ($34 \pm 7\%$; mean $\pm$ SEM for six experiments). In contrast, N-PrP3F4 cells treated with STI1 showed no sign of internalization (Fig. 2E) ($2 \pm 2\%$; mean $\pm$ SEM of four experiments). Thus, the combination of live cell, flow cytometry, immunofluorescence, and biotinylation experiments showed that STI1 was able to induce PrP^C endocytosis in two distinct neuronal cell lines.

Incubation of PrP-null CF-10 cells with STI1 induced neither

Figure 5. STI1 is internalized independently of PrP^C. **A–E**, Wild-type *Pmp*^{+/+} (**A**, **C**); PrP^C-null, *Pmp*^{0/0} primary hippocampal neurons (**B**), CF-10 and CF-10 PrP3F4 cells (**D**); or SN56 cells (**E**) were incubated with 1 μ M STI1–AF568 or STI1 $_{\Delta 230-245}$ –AF568, as indicated, for 40 min at 37°C. Optical sections of representative cells from at least three independent experiments done in multiple cultures are shown. Scale bars, 20 μ m.

PKA nor ERK1/2 activation, consistent with dependence on PrP^C for STI1-dependent signaling, although the cells responded to forskolin (Fig. 3A). The expression of either the wild type (PrP3F4) or the internalization defective PrP^C (N-PrP3F4) reconstituted STI1-induced PKA activation (Fig. 3A). In contrast, transient activation of ERK1/2 by STI1 was rescued in CF-10 by wild-type PrP^C, but not by the mutated PrP^C, which lacks internalization signals (Fig. 3B). The STI1 deletion mutant defective for the PrP^C binding site, STI1 $_{\Delta 230-245}$, is unable to activate either PKA or ERK1/2 signaling in hippocampal neurons (Lopes et al., 2005). In agreement with those observations, CF-10 cells expressing PrP3F4 or N-PrP3F4 presented no activation of ERK1/2 when treated with STI1 $_{\Delta 230-245}$ (Fig. 3C).

Our recent data demonstrated that, similar to chaperones such as Hsp70, STI1 is secreted by astrocytes and presents neurotrophic activity (Lima et al., 2007). To test whether recombinant STI1 may mimic physiologically secreted STI1, we first estimated the amount of STI1 present in CM from astrocytes. Comparison of STI1 in CM with standards containing several concentrations of recombinant STI1 indicates that the amount of STI1 therein is equivalent to ~ 200 ng of protein (Fig. 4A), a STI1 final concentration of 33 ng/ml or 0.5 nM (see Materials and Methods for details). As shown previously (Lima et al., 2007), STI1 from the CM of astrocytes (Fig. 4B, lane 1) can be immunoprecipitated with specific antibodies (Fig. 4B, lane 3) resulting in a fraction of CM depleted of this protein (Fig. 4B, lane 2). The CM containing STI1 at a final concentration of 5 nM was able to induce ERK1/2 activation in PrP3F4-expressing cells, but not in the PrP-null parent CF-10 cells (Fig. 4C, left). Conversely, STI1-depleted CM did not activate ERK1/2 in any of the cell lines (Fig. 4C, right), suggesting that recombinant STI1 reproduces the effects of secreted protein found in CM derived from astrocytes. It should be noted that 100 times more recombinant STI1 (0.5 μ M) (Fig. 3B) than astrocyte-secreted STI1 (5 nM) (Fig. 4C) is necessary to activate ERK1/2. This indicates either that some of the recombinant protein may not be properly folded or that posttranslational

Figure 6. A fraction of internalized STI1 colocalizes with clathrin. PrP3F4 or SN56 cells, as indicated, were transfected with clathrin-GFP and incubated with 1 μ M STI1-AF594 or STI1-AF568 for various periods of time. **A, B**, Representative images for the first minutes after incubation with STI1. Arrows point to some vesicles where clathrin-GFP and fluorescent STI1 are found. **C, D**, Representative images of transfected cells 20 min after incubation with STI1. Right panels present a magnified view of the region indicated in the merged images. Arrows indicate some of the colocalization spots. Images are representative of at least 30 cells from several culture dishes on 3 different days. Scale bars, 20 μ m.

modifications in secreted STI1 as well as the presence of coactivators in the CM may contribute for this higher activity compared with the recombinant protein.

STI1 binds to cells in a specific manner and is internalized

To evaluate STI1 interaction with the cell membrane and its possible dependency on expression of PrP^C at the cell surface, recombinant STI1 or the deletion mutant unable to bind PrP^C, STI1 $_{\Delta 230-245}$ (Lopes et al., 2005), were labeled using AF488, AF568, or AF594. Control experiments showed that labeling STI1 as well as STI1 $_{\Delta 230-245}$ did not produce any degradation (supplemental Fig. 1A,B, available at www.jneurosci.org as supplemental material) and labeled proteins are able to evoke PKA activity in cultured hippocampal neurons (supplemental Fig. 1C, available at www.jneurosci.org as supplemental material) and are therefore functional. Interestingly, STI1-AF568 bound to hippocampal neurons from both wild-type (*Prnp*^{+/+}) (Fig. 5A) and PrP^C-

null (*Prnp*^{0/0}) (Fig. 5B) mice was internalized. In agreement with these observations, STI1 $_{\Delta 230-245}$ -AF568 was also effectively internalized by wild-type neurons (Fig. 5C). These observations were confirmed in PrP-null CF-10 cells and CF-10 cells expressing PrP3F4 treated with STI1-AF488 (Fig. 5D) and also in SN56 cells exposed to STI1-AF568 or STI1 $_{\Delta 230-245}$ -AF568 (Fig. 5E). Therefore, these data suggest that STI1 binds to neuronal cells and is internalized by a PrP^C-independent pathway.

Intracellular localization of internalized STI1

Although the interaction of STI1 with cells appears to be independent of the presence of PrP^C, signaling is strictly dependent on PrP^C expression, and ERK1/2 activation depends on PrP^C internalization. Hence, it is possible that engagement of these proteins occurs at the cell surface and continues in intracellular organelles to activate ERK1/2. We investigated this possibility by examining the localization of fluorescent STI1. Confocal images of CF-10 PrP3F4 cells or SN56 cells showed that we could clearly detect internalized fluorescent STI1 after 10 min of incubation, and additional exposure to medium containing the fluorescent protein lead to increased intracellular accumulation in vesicles (supplemental Fig. 2A,B, available at www.jneurosci.org as supplemental material). The interaction of STI1-AF568 with cells and its sequestration in intracellular organelles showed specificity, because no internalization of STI1-Alexa Fluor was detected in experiments done at 4°C (data not shown). Moreover, intracellular labeling with fluorescent STI1 was blocked when CF-10 PrP3F4 or SN56 cells (supplemental Fig. 2C,D, available at www.jneurosci.org as supplemental material) were incubated with a 10-fold excess of nonfluorescent protein (STI1) for 1 h, followed by incubation with fluorescent STI1.

To test whether fluorescent STI1 is present in the same organelles as PrP^C, we did a series of double-labeling experiments in living cells using GFP-tagged markers of internalization pathways. We chose to study trafficking in living cells to avoid possible fixation artifacts, because GPI-anchored proteins such as PrP^C may change location after fixation, and we noted that fixed STI1-AF568-labeled cells showed a distinct pattern of localization compared with live cells (data not shown). Cells were transfected with clathrin light chain-GFP, which labels both coated pits and clathrin-coated vesicles (Gaidarov et al., 1999). These experiments indicated that STI1 (AF568 or AF594) internalized in cells in the initial period (<15 min) after incubation showed some colocalization with GFP-clathrin-labeled vesicles both in CF-10 PrP3F4 cells (Fig. 6A) and in SN56 cells (Fig. 6B). In contrast, incubation of STI1-AF568 for 20 or 40 min (data not shown) with CF-10 PrP3F4 cells (Fig. 6C) or SN56 cells (Fig. 6D) showed

no colocalization of internalized STI1 (red) with clathrin-coated vesicles (green). Quantification of these colocalization experiments indicated that $41 \pm 8\%$ of STI1-positive vesicles also show clathrin labeling in the initial periods of incubation, but this colocalization decreased to $9 \pm 1\%$ after cells were exposed to fluorescent STI1 for >20 min.

We also investigated a role of raft-derived organelles in the trafficking of STI1, by using either caveolin-GFP (Fig. 7*A*) or flotillin-GFP (Fig. 7*B*) in CF-10 PrP3F4 cells and in SN56 cells (Fig. 7*C,D*). Strikingly, STI1-AF568 (red) partly colocalized with caveolin-1-GFP (green; $36 \pm 8\%$ of colocalization in CF-10 PrP3F4 cells in the initial periods of incubation), but quantification of colocalization after additional periods of incubation in CF-10 PrP3F4 cells showed decreased colocalization ($15 \pm 2\%$ after 20 min incubation). In contrast, STI1-AF568 or STI1-AF594 (red) showed extensive colocalization with flotillin1-GFP (green)-labeled vesicles in all time periods examined ($61 \pm 5\%$ in the initial 15 min incubation and $68 \pm 2\%$ after the 20 min incubation). In SN56 cells, fluorescent STI1 appeared to colocalize extensively with both caveolin- and flotillin-labeled vesicles.

These experiments suggest that internalized STI1 is found predominantly in flotillin organelles at steady state, but a fraction of the protein appears to use clathrin-coated vesicles for internalization, a pathway that also serves as the port of entry for PrP^C (Shyng et al., 1994, 1995; Sunyach et al., 2003; Taylor et al., 2005). To further understand the subcellular localization of STI1, we focused our experiments in SN56 cells, a cell line in which we have previously examined the trafficking of PrP^C and PrP^{res} (Lee et al., 2001; Magalhães et al., 2002, 2005). In agreement with an important role for a raft-derived organelle in the steady-state localization of STI1, we detected almost no colocalization between STI1-AF568 (red) incubated for 20 min (data not shown) or 40 min with AF488-labeled transferrin (green) (Fig. 8*A*) or GFP-Rab5 (green) (Fig. 8*B*), which are markers of classical early endosomes derived from clathrin-coated vesicles.

The above experiments suggest that STI1 may enter cells by two pathways: a smaller fraction of the fluorescent protein seems to use clathrin-mediated endocytosis, but a larger fraction uses a raft-mediated pathway (flotillin and perhaps caveolae in cells that endogenously express caveolin) for internalization. Proteins that are internalized by raft-mediated pathways are able to accumulate in acidic-late endosomal organelles, and flotillin1 (also known as reggie-2) itself is found also in acidic organelles (Sturmer et al., 2004; Pimpinelli et al., 2005). We tested the possibility that STI1 may be located in late endosomes by labeling STI1-AF568-treated cells (1 h) with lysosensor green, a marker of acidic vesicles. Figure 8*C* shows abundant colocalization between STI1-

Figure 7. STI1 is also found in raft-derived vesicles. *A, B*, PrP3F4 cells were transfected with caveolin-1-GFP (*A*) or flotillin1-GFP (*B*), and living cells were perfused with STI1-AF594 for 30 min at 37°C. These images are representative examples of colocalization patterns in the first 15 min of incubation with STI1. *C, D*, SN56 cells were transfected with caveolin-1-GFP (*C*) or flotillin1-GFP (*D*) and incubated with STI1-AF568 for 40 min at 37°C. Insets show a magnified view of the designated box. Colocalization between STI1 and the two markers is seen in yellow in the superimposed images, and arrows show some of the colocalization spots. Images are representative of at least 40 cells from several dishes analyzed on 5 different days for each condition. Scale bars, 20 μ m.

AF568 (red) and lysosensor green (green) in SN56 cells. We confirmed that STI1-AF568 was present in late endosomes/lysosomes by expressing GFP-RAB7Q67L, a constitutively active mutant that is preferentially located in these organelles (Bucci et al., 2000; Magalhães et al., 2005). STI1-AF568 (Fig. 8*D*, red) also colocalized with vesicles labeled with GFP-RAB7Q67L (green). Together, these observations suggest that a larger fraction of fluorescent STI1 accumulates in late endosomes.

STI1 interacts with PrP^C predominantly at the cell surface and in early endocytic intermediates

The above experiments suggest only a small fraction of fluorescent STI1 locates to organelles where PrP^C has been previously found in neurons and neuronal cells (i.e., clathrin-coated vesicles) (Sunyach et al., 2003). To also test in living cells whether both STI and PrP^C can be found in the same organelles, we used STI1-AF568 and GFP-PrP^C. Figure 9*A* (top) indicates that dur-

Figure 8. Subcellular localization of STI1. **A**, SN56 cells were double labeled with STI1–AF568 and transferrin–AF488 for 40 min at 37°C. **B**, Cells expressing GFP–Rab5 were incubated with STI1–AF568 for 40 min at 37°C. Right panels present a magnified view of the region indicated in the merged images. **C**, SN56 cells were double labeled with STI1–AF568 and the acidotropic probe LysoSensor Green (first panel) for 60 min. **D**, Cells expressing constitutive active mutant GFP–Rab7Q67L were incubated with STI1–AF568 for 40 min at 37°C (second panel). The merged image shows colocalizations in yellow. The images are representative of three experiments with multiple dishes. At least 30 cells were analyzed in each condition. Scale bars, 20 μ m.

ing the initial periods of incubation (14 min), a small fraction of STI1 and GFP–PrP^C can be found in similar organelles. Further incubation shows much less colocalization between these two fluorescent proteins (Fig. 9A, bottom).

We have previously demonstrated that GFP–PrP^C localization is affected by a dominant-negative mutant of dynamin I, dynamin K44A (Magalhães et al., 2002). We thus tested whether STI1 internalization would also be altered by dynamin I K44A. In this condition, but not in cells transfected with wild-type dynamin I, STI1–AF568 accumulated at the cell surface in spots (Fig. 9B). Quantification of internalized STI1–AF568 fluorescence suggests that cells transfected with the mutant, but not with wild-type dynamin I, sequestered <5% of the normal fluorescence found in nontransfected cells, whereas dynamin I had no significant effect in the internalization (Fig. 9C). In addition, we found that cells transfected with GFP–PrP^C and treated with STI1–AF568 showed almost no internalization of PrP^C when they were cotransfected with dynamin I K44A (Fig. 9D). Additionally, the decrease in endocytosis by this manipulation caused accumulation of STI1–AF568 at the cell surface in areas where GFP–PrP^C was also concentrated (Fig. 9D, merge). These results suggest that both proteins use a dynamin-dependent pathway for internaliza-

tion and that block of endocytosis causes STI1 to remain bound to PrP^C at the cell surface.

Discussion

The present experiments allowed for the first time the direct visualization of the consequences of engagement between STI1 and PrP^C in living neuronal cells. We revealed that STI1, a PrP^C ligand that causes cellular signaling with consequences for neuronal survival and differentiation, triggers PrP^C sequestration and that this is critical for the activation of ERK1/2 but not PKA. We also noted that STI1 is internalized by cells independently of PrP^C and that although part of STI1 may be internalized together with PrP^C, the two proteins follow distinct downstream intracellular pathways. Finally, signaling by ERK1/2 induced by STI1 is fast and depends on endocytosis of PrP^C, but the interaction of the latter two proteins is likely to be transient once they are both internalized. These results have broad implications for understanding physiological functions of STI1–PrP^C interaction and provide information on how PrP^C trafficking can be affected by stimuli that promote PrP^C-dependent cell signaling.

Induction of heat shock proteins (HSPs) was initially described as an adaptive response that enhances the survival of cells exposed to environmental insults (Parsell and Lindquist, 1993). The evolutionarily conserved STI1, including its human homolog HSP70/HSP90 organizing protein (Hop), interacts with both HSP70 and HSP90 to facilitate the transfer of substrates, thus playing an important role in proper protein folding and maturation (Hernandez et al., 2002). The HSPs are abundantly expressed in both the cytoplasm and the nucleus (Honore et al., 1992; Lasse et al., 1997); however, growing evidence suggest that some HSPs, in particular HSP90 and HSP70, can be secreted by distinct cells (Eustace and Jay, 2004; Evdonin et al., 2006), including astrocytes (Guzhova et al., 2001; Robinson et al., 2005). Secreted HSP70 has been related to prevention of axotomy-induced death of spinal sensory and motor neurons (Houenou et al., 1996; Tidwell et al., 2004), as well as protection against light damage of photoreceptors (Yu et al., 2001) and enhancement of motoneuron survival *in vivo* during the period of naturally occurring programmed cell death (Robinson et al., 2005). Thus, HSP70 may function as a neurotrophic factor.

STI1 binds tightly to HSP70 and might be secreted similarly to the latter, although mechanisms of secretion for both proteins are poorly understood. STI1 is secreted from fibrosarcoma cells (Eustace and Jay, 2004), and we have recently found that the protein is also secreted from primary astrocyte cultures and engages PrP^C-dependent neuronal survival (Lima et al., 2007). Moreover, unbiased proteomic analysis of proteins secreted from cells has detected STI1 and HSPs, which are likely secreted by nonconventional pathways (Keller et al., 2008). Indeed, the recombinant

Figure 9. STI1 interacts with PrP^C predominantly at the cell surface. **A**, SN56 cells expressing GFP-PrP^C (green) were treated with 1 μ M STI1-AF568 (red) for distinct periods of time at 37°C. Images are representative five independent experiments with multiple dishes in which 40 and 12 cells were analyzed. Arrows indicate colocalization spots between GFP-PrP^C (green) and STI1 (red) in forming vesicles. **B**, SN56 cells were cotransfected with the constructs GFP and dynamin I K44A (top row) or wild-type (WT) dynamin I (bottom row), and living cells were incubated with 1 μ M STI1-AF568 for 40 min at 37°C. **C**, Quantitative analysis of the STI1-AF568 internalization in the presence of dynamin I K44A. Quantification was done using ImageJ software (control, $n = 27$ cells; dynamin I, $n = 67$ cells; dynamin I K44A, $n = 85$ cells) by scoring every cell for the total values of fluorescence internalized. The results are the mean values of fluorescence per cell from multiple dishes examined on 3 different days. Error bars represent SEM. For these experiments, a Kruskal-Wallis one-way ANOVA was used ($H = 120$; $p < 0.001$) followed by a Dunn's *post hoc* test ($*p < 0.05$). A.U., Arbitrary units. **D**, SN56 cells coexpressing GFP-PrP^C and dynamin I K44A were treated with 1 μ M STI1-AF568 at 37°C. Z-series were acquired before (GFP-PrP^C) and 40 min after (second to fourth panels) treatment with STI1. Images are representative of four independent experiments in which 15 cells were analyzed. Scale bars, 20 μ m.

STI1 used in the current experiments mimics the extracellular secreted STI1 and induced PrP^C-mediated signal transduction, as expected, for a neurotrophic factor.

It is well known that the association between neurotrophic factors and cell-surface receptors induces diverse cellular signals. Some are triggered as the ligand binds to its cognate receptor at the cell surface, and others depend on the internalization of the receptor-ligand complex, a process known as endosomal signal transduction (Gonzalez-Gaitan, 2003). Indeed, evaluation of STI1 intracellular trafficking as well as its role on PrP^C internalization is of particular interest to understand cell signaling associated with the neurotrophic functions mediated by PrP^C-STI1 interaction. Previously, endocytosis of PrP^C had been linked to a pro-apoptotic activity mediated by p53 (Sunyach and Checler, 2005), but agonists that activate this process are unknown. Recently, it has been shown that signaling derived from STI1-PrP^C interactions and associated with proliferation of glioblastoma cells depends on general cellular endocytic activity (Americo et al., 2007; Erlich et al., 2007). The present study identifies a mechanism by which PrP^C is internalized and revealed that the endocytosis of PrP^C is a determinant of STI1-induced cellular signaling. These observations raise the possibility that other events of PrP^C-mediated signaling, such as those elicited by interaction with neural cell adhesion molecule (Santucci et al., 2005) or antibody cross-linking of PrP^C (Mouillet-Richard et al., 2000; Stuermer et al., 2004), may also depend on the endocytosis of PrP^C. Recent observations have determined that hemin also interacts with PrP^C to induce endocytosis (Lee et al., 2007).

In addition to the downregulation of cell signaling (Drake et al., 2006), endocytosis of cell-surface receptors provides for the activity of signaling endosomes (Luttrell et al., 1999; Lefkowitz and Whalen, 2004). Similar to the dichotomous nature of signal transduction by β 2-adrenergic receptors (Daaka et al., 1998), activation of PKA appears to be independent of the endocytosis of PrP^C, whereas ERK1/2 appears to be highly dependent on PrP^C internalization. ERK1/2 activation mediated by PrP^C-STI1 interaction is known to promote neuronal differentiation (Lopes et al., 2005) and synaptic plasticity (Lopes et al., 2005; Coitinho et al., 2007). Further details on the mechanism of activation of the ERK pathway by STI1 should help clarify this biologically relevant neurotrophic role of PrP^C.

Our data revealed that extracellular STI1 is also internalized by endocytosis. The site that STI1 binds for its entry in cells has not been identified, but the experiments with the mutant STI1_{Δ230–245} and with *Prnp*^{0/0} neurons conclusively discarded the need of PrP^C for STI1 entry in cells. Colocalization experiments implicate lipid raft-derived organelles in the intracellular trafficking of STI1. Although we found that a small fraction of fluorescent STI1 colocalizes with clathrin-coated vesicles, STI1 was not found in classical early endosomes in living cells. In contrast, a significant portion of the protein was found in flotillin-derived vesicles. Flotillin1-positive vesicles were proposed to differ from caveolae-derived vesicles (Glebov et al., 2006), but both likely originate from lipid raft-derived regions of the membrane (Harder and Simons, 1997). In agreement with these results, fluorescent STI1 also accumulates in acidic organelles that recruit GFP-Rab7, a marker of late endosomes/lysosomes. In this aspect, internalized STI1 presents a remarkable similarity to fluorescent PrP^{res}, which was recently shown to be sequestered in SN56 cells in Rab7-positive acidic organelles (Magalhães et al., 2005).

Intracellular trafficking of GPI-anchored proteins is complex, and these proteins can follow distinct pathways depending on the cell type (Fivaz et al., 2002). Despite reports suggesting internalization of PrP^C via caveolae (Peters et al., 2003), overwhelming evidence favors a model of endocytosis of PrP^C via clathrin-coated vesicles (Shyng et al., 1994; Sunyach et al., 2003; Taylor et al., 2005). Recently, an essential role in the endocytosis of PrP^C has been ascribed to the low-density lipoprotein receptor-like protein (LRP1) (Morris et al., 2006; Taylor and Hooper, 2006; Parkyn et al., 2008), by allowing PrP^C to enter clathrin-coated vesicles. In addition, LRP1 appears to have a role in the surface trafficking of PrP^C (Parkyn et al., 2008). Dynamin I dominant-negative mutant K44A blocks both clathrin-mediated endocytosis and caveolae-mediated endocytosis (Henley et al., 1998; Nichols and Lippincott-Schwartz, 2001; Conner and Schmid, 2003). We found that dynamin I K44A suppressed the endocytosis of both STI1 and PrP^C. Furthermore, the use of dynamin I K44A revealed that interaction of STI1 and PrP^C at the cell surface has a transient nature, but inhibition of endocytosis allowed the identification of an intermediate step in which the two proteins appear to remain together.

A likely scenario to explain these results is that STI1 binds to PrP^C at the cell surface and this triggers the interaction of the complex with unknown plasma membrane components that may mediate signaling by PKA. Simultaneously, PrP^C endocytosis is triggered, and that may lead to a new set of interactions, perhaps involving LRP1 or other proteins that can help PrP^C to piggyback to clathrin-coated vesicles, which may be important to cause ERK1/2 activation. Indeed, LRP1 has been shown to link cellular activation to ERK signaling (Orr et al., 2003) and is therefore a likely candidate to participate in this process for STI1–PrP^C. Thereafter, STI1 and PrP^C appear to follow parallel pathways downstream into the cells.

The physiological roles for STI1–PrP^C signaling *in vivo* remain to be established (see (Linden et al., 2008)). However, it is of remarkable interest that expression of truncated forms of PrP^C in a PrP-null background has been consistently shown to cause neurodegeneration in transgenic mice (Shmerling et al., 1998; Baumann et al., 2007; Li et al., 2007). This intriguing phenotype resembles some of the alterations in prion disorders and can be mitigated by expression of wild-type PrP^C in transgenic mice (Shmerling et al., 1998; Baumann et al., 2007; Li et al., 2007). The binding site of STI1 onto PrP^C (amino acids 113–128) (Zanata et al., 2002) is lacking in all truncated proteins that produce this

phenotype (Shmerling et al., 1998; Baumann et al., 2007), including a recently described mouse line with a deletion in amino acids 105–125 of PrP^C (Li et al., 2007). Future experiments should unravel whether the mechanisms described here for STI1-mediated PrP^C signaling contribute to such phenotypes.

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